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14. ABSTRACT

The functional importance of gene enhancers in regulated gene expression is well established and recent evidence indicates that, in addition to widespread transcription of long non-coding RNA (ncRNA) transcripts in mammalian cells, bidirectional ncRNAs, referred to as eRNAs, are present on enhancer, However, it has remained unclear whether these eRNAs are functional, or merely a reflection of enhancer activation and may be a cause of upregulation of cancer genes. We found that breast cancer causing,17β-estradiol (E_2)-bound estrogen receptor α(ERα) on enhancers causes a global upregulation of eRNA transcription in enhancers adjacent to E_2 upregulated coding genes in MCF-7 (Breast cancer cells). These induced eRNAs are required for the observed induction of target coding genes, causing the increased strength of specific enhancer:promoter looping, Our data indicate that eRNAs induced by liganded ERα are required for effective recruitment of critical machinery required for estrogen regulated coding gene activation events and suggest that eRNAs are likely to exert important functions inmany regulated programs of gene expression in Breast cancer progression.

15. SUBJECT TERMS

E2–17β-estradiol

ERα – Estrogen receptor alpha

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Table of Contents

Page

Dissecting an estrogen receptor-regulated enhancer code in breast cancer

Introduction

Breast cancer is the most common cancer in women in the western world and it is estimated that there will be more than 40,000 deaths from breast cancer in the United States alone this year (1). Despite years of intense investigation, the treatment is far from optimal and the underlying molecular mechanisms causing this cancer are not yet fully understood. Estrogen receptor alpha (ERa), found in 2/3 of breast cancer at diagnosis, belongs to nuclear receptor super-family and functions as a transcription factor mediating estrogen response. Several recent discoveries have greatly altered our current views of regulated gene transcription, with the growing realization that alterations in nuclear architecture, non-coding RNAs and activation of DNA repeats, have important implications for breast cancer. Recent data in many species and cell types are beginning to reveal that cellular identity is set primarily by the cohorts of enhancers unique to that cell-type and which constitute 50-70% of the cellular enhancers. Based on global genomic data, it is now possible to sub-classify these enhancers into different cohorts, each of which exhibit different GO terms, implying that they regulate distinct functions in the breast cancer cells. Intriguingly, the vast majority of ERa binding sites are primarily located in intergenic and intronic regions, including enhancer regions (2,3,4), that activate/repress target gene transcription. This eventually leads to activation/repression of transcriptional programs involved in cancer pathogenesis, provoking the hypothesis that by regulating different functional gene cohorts, there would be a rational approach to screening for new therapeutic agents that selectively affect specific portions of the tumor transcriptome that contribute to resistance and progression. This necessitates our identification of those enhancers that actually regulate specific gene transcription, and development of an open-ended method to determine the precise, estrogen-induced programs of enhancer:promoter interactions, and the roles of enhancer-encoded ncRNAs.

Proposed proposal had two aims:

Specific Aim 1: To classify the precise, global interactions of ERa-regulated gene enhancers with all target genes, in the presence and absence of E2, using the 3D-DASL strategy, and to define the dynamic alterations in the enhancer:promoter programs and transcriptional activation programs revealed using RNA-Seq and global run-on (GRO-Seq) approaches, that underlie activation/repression of distinct breast cell enhancer cohorts with respect to "programming" of transcriptional response in normal breast and tumor behavior.

Progress: In last year progress report I had shown the data obtained form 3D-DSL experiments, where I had taken 20 E2 (Estradiol)-target genes and performed chromatin architecture assay, I found that interaction betIen enhancer-promoter induces many folds upon E2 treatment further the eRNA knockdown completely abolished this interaction. HoIver, the Key question remains whether eRNA (Non-coding RNA) transcribed from ERa-regulated enhancer is functional or is it a by-product of enhancer activation?

E2-regulated enhancers generally displayed a basal expression of bidirectional eRNAs and, these are in proximity to up-regulated coding genes displayed a characteristic bidirectional activation of eRNAs, The eRNA transcripts vary in apparent length but Ire generally ~1.5 kb, although ~10% exhibited an apparent predominance of unidirectional eRNA transcripts. Analysis of the GRO-seq data confirmed the overall upregulation of eRNAs in response to ligand, generally with bidirectional transcription, robust at 1h after E2, and subsequently diminishing, being highly diminished by 24h. Where the depth of sequencing permitted clear analysis of expression of the eRNA, at least one enhancer adjacent to E2/ERα up-regulated coding genes invariantly exhibited E2-dependent up-regulation of their eRNA; hoIver for some of the remaining upregulated coding transcription units, the eRNA expression levels Ire below those required for accurate quantitation. Overall, >83% of ERα-bound enhancers adjacent to up-regulated coding genes exhibited E2-induced eRNA transcription by GRO-seq. The median distance betIen enhancers exhibiting E2-dependent up-regulation of their eRNAs and their closest up-regulated coding gene was ~52kb, with most <215 kb from the coding gene cap site, compared with a median distance of >270kb for enhancers exhibiting ligand-insensitive enhancer eRNAs with corresponding non-responsive coding genes. Examining the strength of ERα binding, based on normalized ChIP-seq data on these cohorts of enhancers with upregulation of the eRNAs, exhibited significantly stronger binding than on enhancers not exhibiting eRNA upregulation.

To validate independently that eRNAs per se are important for quantitative increases in target gene expression, I took advantage of a GAL4–BoxB-tethering-based reporter assay (5). For this I engineered a chimaeric RNA by fusing FOXC1 sense eRNA to BoxB viral RNA, permitting BoxB-FOXC1 eRNA to be recruited by the RNA binding domain of lN protein fused with the GAL4 DNA binding domain (lN–GAL4). Thus eRNA can be artificially tethered to 53UAS sites just downstream of the FOXC1 enhancer in the reporter plasmid, in which luciferase (Luc) is under the control of the native FOXC1 promoter (Fig. 1). I observed that the presence of full-length FOXC1 enhancer increased Luc expression to, 2.5 fold when compared to random DNA in place of the enhancer

(Fig. 2a, blue bars). This effect was abolished when the sense eRNA sequence was deleted and substituted with 5XUAS sites, generating a non-functional 'missense' eRNA (Fig. 1a, blue bars, and Fig. 2). Tethering of BoxB-FOXC1 eRNA, but not BoxB alone, could fully rescue the activity of loss of sense-eRNA-deleted enhancer (Fig. 2a, orange bars), whereas the antisense FOXC1 eRNA could not (Fig. 2b). I confirmed the loss of plasmid-driven native FOXC1 eRNA expression from the sense eRNA- deleted construct.

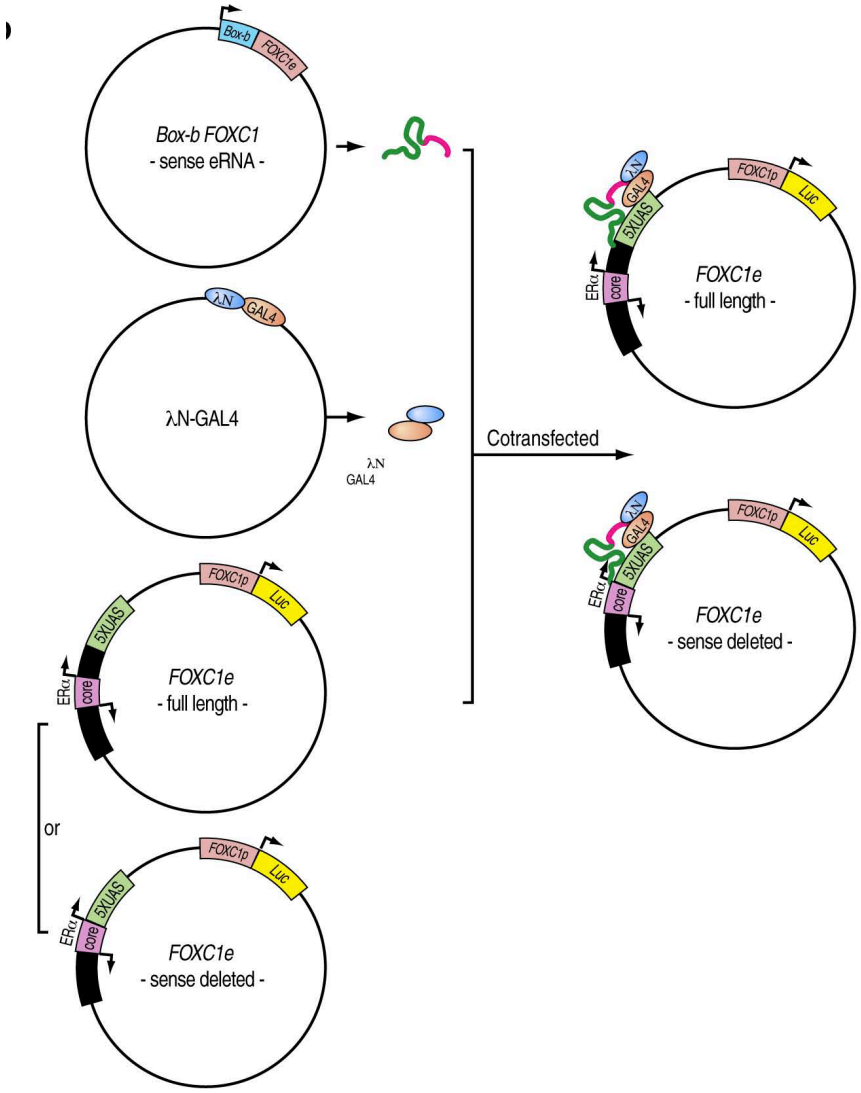

Fig1. The procedures of Box-b tethering assay. (a) panel showing the diagram of sense-eRNA deletion strategy where 5XUAS sites recruit GAL4-λN along with Box-b-FOXC1e eRNA on a truncated enhancer site.

plasmid-driven native FOXC1 eRNA expression from the sense eRNA- deleted reporter construct, and showed that GAL4 tethering was not altered (Fig. 2c, d). These data further support the suggestion that the sequence-specific eRNA transcript per se, rather than merely the process of enhancer transcription, is required for the actions of the eRNA on enhancer-dependent coding-gene activation events. This observation is consistent with

recent studies of the role of ncRNAs in p53-dependent gene activation (6) and in regulation of the SNAI1gene(7).

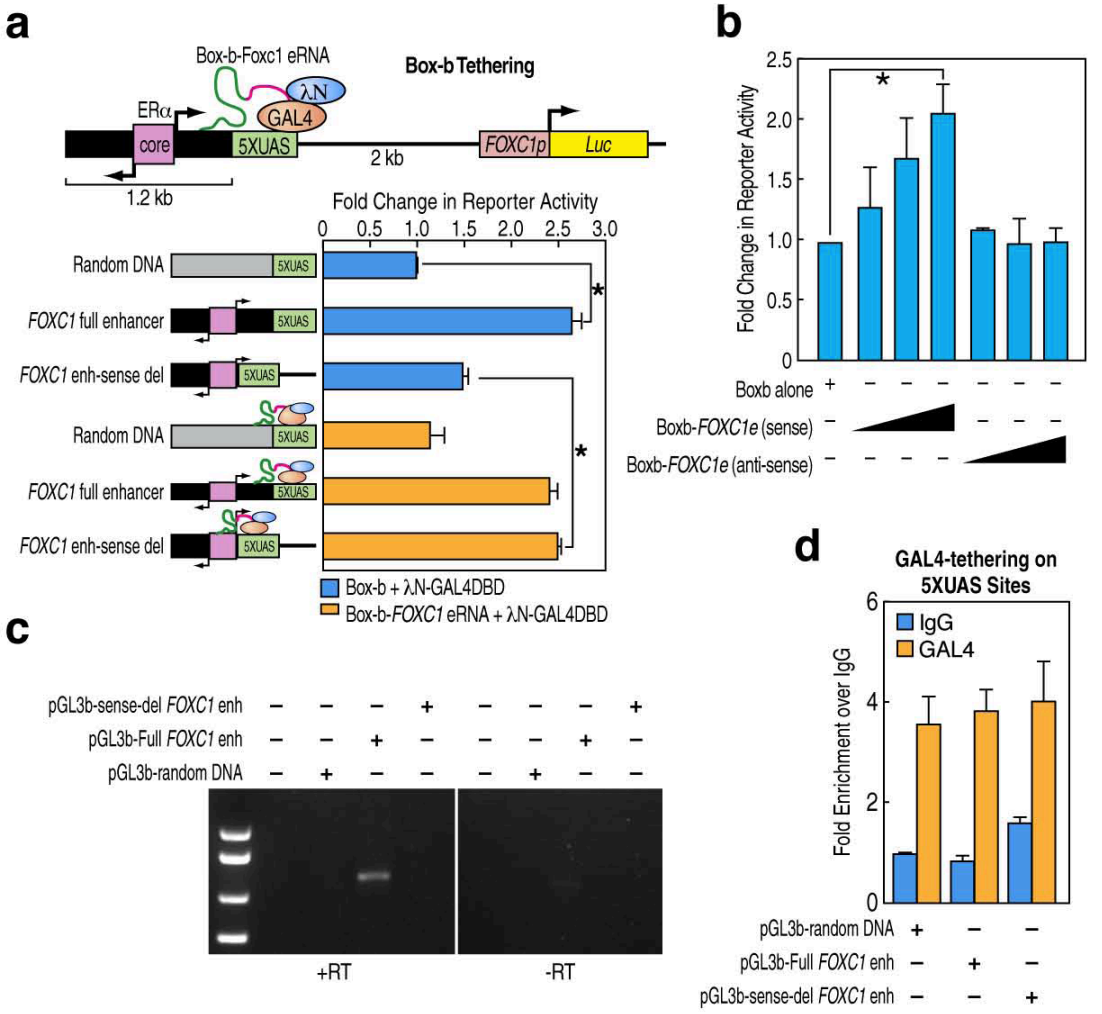

Fig2. Ligand-induced eRNA is functionally important. (a) Schematic diagram of the Box-b-λN tethering system on *FOXC1* enhancer which is upstream of a *FOXC1* native promoter-linked luciferase cassette. 5XUAS sites are fused downstream to *FOXC1* enhancer. GAL4-λN fusion product tethers *Box-b-FOXC1* eRNA to the 5XUAS sites. Bar graph shows the effects of the *FOXC1* eRNA on *FOXC1* promoter-driven luciferase activity in the presence of E_2 (24 hrs). **Blue bars:** the activating function of the native full length enhancer (Bar:2) over random DNA (Bar:1) is lost when sense eRNA cassette is substituted with 5XUAS site, which generates mis-sense eRNA (Bar:3). **Orange bars:** The loss of enhancer-mediated activation was largely rescued when the *FOXC1* eRNA is tethered to the mis-sense eRNA transcribing enhancer (Bar 6). **(b)** eRNA function is sequence-specific: *FOXC1e* sense eRNA but not anti-sense strand (- strand) eRNA could rescue the activity of mis-sense eRNA transcribing enhancer, in the Box-b tethering assay. (**c**) Gel picture showing plasmid-based eRNA expression from full length enhancer construct but not from truncated enhancer construct. **(d)** Bar graph showing efficiency of GAL4 tethering on various pGL3b constructs**.**

LNA mediated knockdown does not cause the transgene silencing: To rule out the possibility of LNA mediated transgene silencing, I hypothesized that if after LNA treatment enhancer undergoes silencing then transcriptional activity should be affected or In other words, since enhancer transcribes eRNA from both sense and anti-sense strand thus in ideal condition, knock down of sense eRNA should not affect the transcription of antisense strand and vice versa but, in case of transgene silencing it should affect not only the transcription both ways but histone H3 on targeted region of enhancer should also udergo the repressive modifications namely; H3k9me3 and H3k27me3.

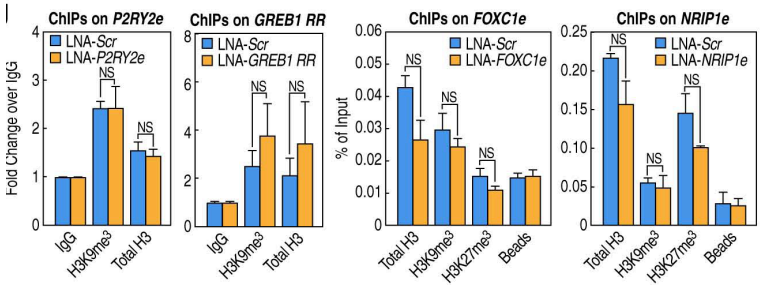

Fig3. LNA/siRNA do not cause transgene silencing on enhancer region: ChIP-QPCR analysis depicts that LNA treatment do not affect the total H3 and H3K9me3 level on P2RY2e and GREB1 RR or total H3, H3K9me3 and H3K27me3 level on FOXC1e, and NRIP1e. Mean±SD. (n=3). *p< 0.05, **p<0.01.

Thus to check this hypothesis, I chose P2RY2, GREB1, FOXC1 and NRIP1 enhancers and selectively knocked down the sense eRNA by using specific-LNA's. MCF-7 cells were transfected with LNA's targeting sense eRNA from all these enhancers. After 24 hrs of transfections, cells were subjected to ChIP with following antibodies; Total histone H3, h3k9me3 and k3k27me3. Q-PCR analysis of resultant chromatin shows there is no significant difference among these modifications and between LNA treated and untreated cells (Fig. 3). Suggesting that LNA's do not cause the transgene silencing and can be used in enhancer therapy where goal will be to modulate the gene expression of any disease causing gene by targeting eRNA on corresponding enhancer.

Specific Aim 2: To perform these analyses on (unidentified) tumor specimens from ERa positive, ERa negative, double negative and metastatic tumors, to begin to translate our findings to individual tumors, as a basis for new diagnostic markers and therapeutic approaches.

Tumor samples for aim 2 have been collected, and 3D-DSL have been performed for 20 genes, data is under analysis to identify new therapeutic approaches.

Experimental Methods:

(1) BoxB-lN tethering assay. Similar to the previous method(5), as described in Fig. 2a and Fig. 1, the BoxB tethering system uses viral RNA–protein interactions, in which

BoxB is a viral RNA that can be recognized and bound by viral anti-terminator protein lN. Fusion of FOXC1 eRNA with BoxB enables the fused BoxB-FOXC1e to be bound by lN. Subsequently, lN protein was fused to the DNA-binding domain (DBD) of GAL4, which then recognizes 53UAS sites on the reporter plasmid DNA. Using this technique, BoxB-eRNA can be tethered to the 5XUAS sites on a reporter plasmid with the help of the lN–GAL4 fusion protein(5). Full-lengthFOXC1 eRNAwas cloned in pCDNA3.1 downstream to five copies of BoxB. This construct was co-transfected along with the reporter plasmids and lN–GAL4 vector (Fig. 1), which is also based on a pCDNA3.1 vector with CMV promoter. FOXC1 promoter was cloned in KpnI and BglII sites in pGL3-basic vector, 5XUAS sites Ire cloned at upstream SalI site in pGL3-basic vector, FOXC1 full-length enhancer (1.2 kb) was placed just upstream to 5XUAS sites at the BamHI site. For deletion of the sense eRNA, the enhancer region was amplified including the full antisense transcript, the core region and 20 nucleotides from the sense eRNA of the FOXC1 enhancer (thus called FOXC1 enh-sense del enhancer, Fig. 2c) was also cloned at BamHI site upstream to 5XUAS site (Fig. 1).

Luciferase reporter assay. Tethered plasmids alone or in combination Ire transfected along with Renilla-TK plasmid into MCF-7 cells that had been hormone stripped for 3 days. Six hours post-transfection, they Ire treated with 10nM E2 for 24 h further, and then they Ire subjected to the luciferase assay using the Dual-Lucifersae reporter assay kit (Promega); plates Ire read in Veritas Microplate Luminometer (Turner Biosystems).

Gro-seq and analysis: GRO-sequencing of nascent RNA was achieved using MCF-7 cells hormone stripped for three days and treated or untreated with 100nM Estradiol, was employed as mentioned in (8).

MCF-7 cells were initially obtained from ATCC. Custom siRNAs were designed in-house and chemically synthesized by Sigma-Aldrich®, while LNAs were designed and synthesized by Exiqon®. Knockdown experiments with either siRNAs or LNAs were performed as transient transfections using Lipofectamin 2000, as per manufacturer's instructions (Invitrogen). For siRNAs, cells were cultured for 2 days after transfection and then treated with either vehicle or E2 for 1 h.; for LNAs, cells were cultured for either 6 or 24 h. after transfection and exposed to the same treatment as described above. Immunoprecipitations and Real-time qPCR was carried out as previously described (9)**,** normalized to either ACTD or GAPDH, with the exception that high throughput sequencing libraries Ire prepared as per Illumina's HiSeq 2000 library reagent kit. Global run-on sequencing experiments (GRO-seq) were performed as previously reported (10).

Key Research Accomplishments:

1. Deep ERa ChIP-sequencing in MCF-7 cells identifies 3000 new ERa bound sites in genome.

2. Nascent Transcription sequencing (Gro-seq) in MCF-7 cells reveals bidirectional RNA transcription on ERa bound enhancers indicating, role of ERa in enhancer mediated gene regulation of cancer causing genes.

3. SiRNA mediated knockdown of these eRNA dysregulates the neighboring gene transcription suggesting these eRNA have important role in estrogen target gene program.

4. 3D-DSL developed in Lab indicates that ERa enhancers physically loop with adjacent promoters and eRNA knockdown diminishes such interactions.

5. Tehered-reporter assays performed, show that eRNA are functionally important for estrogen-dependent gene regulation.

Reportable outcomes:

Based on above key research accomplishments, a manuscript describing the role of ERa bound enhancers in gene regulation is published, where I am sharing first authorfhip. Title of manuscript is

> Li W*, Notani D*, Ma Q, Tanasa B, Nunez E, Chen AY, et al. Functional roles of enhancer RNAs for oestrogen-dependent transcriptional activation. Nature. 2013 Jun 27;498(7455):516-20. PMID: 23728302.
>
> * Equal contribution

Conclusions:

Despite the discovery of enhancers more than 35 years ago, full understanding of the biology of their generation, and the mechanisms by which they regulate gene expression has been a relatively resistant problem. Our finding that the induction of enhancer non-coding RNAs, often as bidirectional transcripts, by Estrogen signaling, posing the question of the functional importance of their regulation, if any. I have provided several lines of evidence that induced eRNAs are functionally important for many roles of estrogen-induced gene enhancers, being necessary for the dynamic generation/stabilization of enhancer:promoter loop activation of the regulated coding transcription units adjacent to these ERα-bound enhancers, and E2-dependent activation of coding gene transcription. I suggest that liganded ERα acts, at least in part, to increase recruitment of the Cohesin complex to enhancers, apparently requiring eRNA expression for stable association with the enhancer. Further, eRNA tethering reporter assay show that eRNA per se are important for gene activation. I infer from these observations that enhancer specificity is derived from binding of the liganded receptor, initiating induction of eRNAs, that may often or invariantly utilize a common machinery, including Cohesin, to strengthen promoter:enhancer interactions and modulate target coding gene activation events.

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